

Metabarcoding to Investigate Changes in Soil Microbial Communities within Forensic Burial Contexts

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Abstract

The estimation of the time elapsed since death (post-mortem interval, or PMI) is one of the key themes that forensic scientists have to address frequently. However, the estimation of PMI still suffers from poor accuracy and biases especially when decomposition stages are prolonged, so further improvements in methods for PMI estimation are desirable. Soil microbial communities associated with decomposing bodies have been shown to be good candidates for the estimation of the PMI of exposed bodies. However, further research is required to better understand the bacterial succession associated with decomposition of buried carcasses, to test its reliability and applicability for the estimation of PMI and to better understand the dynamics involved with decomposition within this particular scenario. Therefore, we explored the succession of soil microbial communities associated with four decomposing pig carcasses (from one to six months PMI) using a metabarcoding approach. The sequencing of the bacterial 16S rRNA variable region 4 (V4) revealed trends linking specific microbial taxa with specific PMIs, and in particular an increase in Proteobacteria, Firmicutes and Bacteroidetes at specific PMIs as

well as a decrease in Acidobacteria after the introduction of the carcass in the soil. Our results, in accordance with previous studies conducted on exposed bodies of different mammalian species (including humans), showed also a general reduction of the soil taxonomic richness from two months PMI onwards, as well as an incomplete re-establishment of the starting soil microbial conditions after six months PMI, with great relevance for forensic investigations. We also found specific mammals-derived taxa, such as *Bacteroides* spp., being still present in the soil after six months PMI. This study serves as a baseline for additional research to allow the characterisation of specific biomarkers associated with specific PMIs. Due to the similarity between the results presented here and those reported in other types of decomposition studies we believe that the metabarcoding approach might have considerable potential in the estimation of the PMI, particularly to clarify cases involving heavily skeletonised bodies or for the investigation of clandestine graves in which the carcass has been moved from its original place of deposition.

Keywords

Post-mortem interval, decomposition, metabarcoding, 16S rRNA, taphonomy.

Introduction

Soil consists of a complicated mixture of mineral materials and decaying organic remains of bacteria, fungi, nematodes, mammals, insects and plants[1], as well as living organisms such as microorganisms, invertebrates and small mammals. Due to its ubiquity, soil has been frequently used in forensic investigations to assess the involvement or not of a suspect based on the important and exclusionary information contained in this complex matrix. Classically, forensic analyses on soil were conducted on its chemical and physical characteristics[2], including particle size[3], soil colour[4], organic content[5], elemental composition[5], mineral abundance[6]. More recently, metagenomic analyses based on the identification of soil microbial communities have been increasingly used ([7–9]) to characterise and discriminate different soils of forensic interest.

Despite the well-known use of molecular approaches to identify different soil types, the application of these techniques to investigate which microbial communities are associated with specific post-mortem intervals (PMIs) of carcasses has been much less explored. Bacteria and fungi share the important role of recycling the organic matter within the soil with their carbon and nitrogen cycles[10,11], and the presence of a decomposing body clearly alters the original balances of species and abundances within the microbial communities. The decomposition rate and pattern of a body on the soil surface or within a grave is a very complex phenomenon that strongly depends on some biotic and abiotic factors[12], and on some intrinsic characteristics of the body (e.g., body size[13–15] or trauma[16,17]). In general, it starts with the autolysis process, in which the accumulation of carbon dioxide in tissues causes the release of enzymes leading to cell death[18,19]. Subsequently, the anaerobic intestinal flora starts to break down various macromolecules like proteins, lipids and carbohydrates, inducing the typical changes in colour and bloating of the carcass[20]. During this stage, fluids present in the body start to purge into the soil due to the internal pressure created by expanding gases, and can induce an important shift in the microbial community present within the gravesoil due to the changes in their original environments and due to the presence of newly released carbon, nitrogen and phosphorus compounds[21]. Changes in the availability of these nutrients within soil may induce the growth of some microbial species and inhibit the presence of other species, directly or indirectly[19]. For this reason, the presence and abundance of microbial species existing in the burial environment may vary sequentially during specific decomposition stages, and their identification may be useful when linked to known PMIs of the carcass. The important factors are likely to be (a) the provision of a new food source for existing soil

biota, (b) the introduction of a source of new microbiota from the cadaver itself, (c) the attraction of invertebrates to the cadaver which will contribute with further microbiota and (d) the cellular DNA and proteins derived from the decomposition of the cadaver and from the colonising invertebrates.

Several attempts have already been made to evaluate the changes in the microbial communities associated with decomposing bodies with different DNA-based strategies. For example, Parkinson *et al.*[19] performed two separate studies in which they investigated changes in the microbial community associated with three human cadavers left exposed on the ground surface for different time periods using the profiling method of T-RFLP analysis of 16S rRNA bacterial genes and of the ITS region on fungal rRNA. Their results showed that a succession of different bacteria and fungi were associated with different decomposition stages, but they were not able to identify individual biomarker species associated with specific decomposition stages due to the limits of the technique used for the study[19].

Alternative methodologies involving the analyses of bacterial or fungal communities through the use of denaturing gradient gel electrophoresis (DGGE) analyses continued to be used even recently, at least when these studies were applied to forensic sciences. Bergmann and Thompson[22] used an alternative RNA-based approach to evaluate changes in the soil bacterial communities at different depths, after a pig leg was buried in sandy soil. They showed, in accordance with results previously reported in the literature, that the richness of the microbial taxa increased from 3 to 28 days and then decreased from 28 to 77 days after the burial, although the limits of the technique used did not provide any helpful indication about taxa associated with specific PMIs[22]. Olakanye *et al.*[23] used the same experimental design as Bergmann and Thompson[22] but their study lasted 14 weeks and was focused on DNA instead of RNA. At selected time points, soil at different depths was collected by coring and the 16s rRNA gene from soil bacteria was analysed through PCR-DGGE. They showed an increase in the species diversity within soil collected from the burial site compared with a control soil between 10 and 71 days; however, the intrinsic limits of the technique chosen for the study did not allow the identification of specific biomarkers useful for the estimation of the time elapsed from the beginning of the burial period[23]. Chimutsa *et al.*[24] focused their study exclusively on fungal community variations to look for cadaver decomposition indicators, using the same experimental design previously adopted by Bergmann *et al.*[22] and Olakanye *et al.*[23]. In contrast with what they found for bacteria, they did not observe any statistically significant

variation in eukaryotic communities comparing the soil collected close to the carcass and the control using the 18S rRNA gene analysis through PCR-DGGE, opening questions about the reliability of this approach when only fungi are analysed[24].

To overcome these limits, new studies using high-throughput DNA sequencing approaches have investigated the microbial community associated with decomposition. Hyde *et al.*[25] collected bacterial swab samples from different internal and external sample sites on two decomposing human cadavers placed outdoors and analysed the samples using 454 pyrosequencing. They noticed changes in the bacterial community associated with specific PMIs, finding bacteria related to the presence of flies during the bloating and purging phase and bacteria related to soil after the skeletonization phase. Pechal *et al.*[12] investigated the “necrobiome” succession on the skin of exposed carcasses of freshly killed swine during five days from their initial exposure. Their bacterial 16S rRNA showed that specific families of bacteria were more abundant after specific PMIs, in addition to the observation of an overall decrease of the taxon richness over the course of the decomposition period. Furthermore, they proposed a model to estimate the time of death, though noting that the model required independent validation[12]. Cobaugh *et al.*[26] explored the bacterial succession beneath four exposed human cadavers during successive decomposition stages, and found that specific phyla were present at specific decay phases up to 198 days post-mortem. Metcalf *et al.*[27] showed in a study of exposed decomposing mouse bodies that the combination of both prokaryote and eukaryotes datasets collected on skin and on gravesoil for 48 days resulted in an accurate estimation of the PMI. In particular, they found specific taxa that may be diagnostic for potential forensic applications, and more data may improve this knowledge to allow a future application of this method (the so-called “microbial clock” method) to the criminal justice system[27]. In a subsequent paper Metcalf *et al.*[28] advanced upon their previous study by studying the bacterial and fungal community assemblies associated with both exposed mouse and human decomposition, also comparing different types of soils. They identified specific networks of decomposers at selected PMIs, and they also highlighted the fact that the three different soil habitats (grass prairie, subalpine forest, and desert environments) did not change the outcome of the results, opening the way to new opportunities in the application of these studies to forensic contexts[28].

Despite the obvious advantages and improvements introduced by the use of NGS approaches, methodologies involving the analyses of bacterial or fungal communities through the use of denaturing gradient gel electrophoresis (DGGE) analyses continued to

be used even recently, at least when these studies were applied to forensic sciences. Bergmann *et al.*[22] used an alternative RNA-based approach to evaluate changes in the soil bacterial communities at different depths, after a pig leg was buried in sandy soil. They showed, in accordance with results previously reported in the literature, that the richness of the microbial taxa increased from 3 to 28 days and then decreased from 28 to 77 days after the burial, although the limits of the technique used did not provide any helpful indication about taxa associated with specific PMIs[22]. Olakanye *et al.*[23] used the same experimental design as Bergmann *et al.*[22] but their study lasted 14 weeks and was focused on DNA instead of RNA. At selected time points, soil at different depths was collected by coring and the 16s rRNA gene from soil bacteria was analysed through PCR-DGGE. They showed an increase in the species diversity within soil collected from the burial site compared with a control soil between 10 and 71 days; however, the intrinsic limits of the technique chosen for the study did not allow the identification of specific biomarkers useful for the estimation of the time elapsed from the beginning of the burial period[23]. Chimutsa *et al.*[24] focused their study exclusively on fungal community variations to look for cadaver decomposition indicators, using the same experimental design previously adopted by Bergmann *et al.*[22] and Olakanye *et al.*[23]. In contrast with what they found for bacteria, they did not observe any statistical variation in eukaryotic communities comparing the soil collected close to the carcass and the control using the 18S rRNA gene analysis through PCR-DGGE, opening questions about the reliability of this approach when only fungi are analysed[24].

Although a limited number of studies have been conducted on this topic, it has been shown that the analysis of the microbial succession within gravesoils may become a useful technique to characterise the decomposition stage and/or the PMI of carcasses even if the body has been removed from its original place of burial. However, significant limitations are still present within this field in particular for subsurface cadaveric decomposition as the majority of these studies have been done on above-ground decomposition[11]. For this reason, there is the need for new research on this topic to investigate changes in the microbial populations when whole carcasses are buried in the ground instead of being exposed on the surface to the action of invertebrate and vertebrate scavengers. Finley *et al.*[29] investigated the microbial succession in gravesoil associated with exposed or buried human corpses, but the buried carcasses were only four (in comparison with fourteen bodies left on the surface to decompose) and the time intervals between soil sampling were relatively long (8, 96, 214 and 303 days), leaving gaps in the

characterisation of microbial successions during the so-called “active decomposition stage” (the putrefaction/liquefaction stage occurring in general during the first three months post-mortem).

Here we explored the microbial succession of the bacterial community within gravesoil after the burial of whole pig cadavers under ~40 cm of soil in outdoor settings for six months, using metabarcoding DNA sequencing approaches targeting a specific gene region (16s rRNA) to overcome some of the limits highlighted above. Together, these data allowed us to investigate the changes that take place within a “closed environment”, such as the burial grave, when a decomposing body disturbs the normal environmental microbial community. We monitored changes associated with different PMIs (and decomposition stages), with the ultimate aim of identifying specific microbial taxa that may be associated with specific stages of decomposition.

Materials and methods

Experimental design

Experimental forensic burials of four juvenile pigs (*Sus scrofa*) were undertaken at the HuddersFIELD outdoor taphonomy facility (University of Huddersfield, U.K.) between May and November 2016. Soil physicochemical and biological characteristics on a control sample taken at ~40 cm depth prior to the burials (performed by the CNR - Institute for the Study of the Ecosystems, Pisa, Italy) resulted in electrical conductivity (0.53 dS m^{-1}), total N (0.448%), total organic C (6.29%), total P (1.95 g/Kg), available P (62.93 mg/kg), NO_3^- (69.84 mg NO_3^- /kg), NH_3 (1.93 mg NH_3 /kg), dehydrogenase activity (0.96 $\mu\text{gINTF/gss}\cdot\text{h}$), β -glucosidase activity (34 $\mu\text{molMUB/g}\cdot\text{h}$), phosphatases activity (31 $\mu\text{molMUB/g}\cdot\text{h}$), arylsulfatase activity (314 $\mu\text{molMUB/g}\cdot\text{h}$), protease activity (15 $\mu\text{molAMC/g}\cdot\text{h}$). Details of the experiment and of the carcasses were reported in Procopio *et al.*[30] and are briefly summarised here. The experiment was conducted following the guidelines of the U.K. Department for Environment, Food and Rural Affairs (DEFRA). The pigs, which were of similar age (3-5 weeks old) and weight (4.5-11 kg), died naturally from unknown causes, were frozen immediately after death at -20°C and were kept frozen until the beginning of the experiment. Due to the nature of the experiment and for ethical and legal reasons, it was difficult to collect animals similar in breeds, size, sex, cause of death, nourishment given *et cetera*, but we did make an effort to limit the variability between the animals as much as possible. Despite being aware that the intestinal biota may vary in relation with

different types of diet[31], we could not have control on this specific variable (since the nature of the work excluded the slaughtering of pigs with controlled diets). However, we managed to obtain pigs at least with similar weights (and hence similar ages-at-death), to minimise the differences in their decomposition rate which are known to exist between carcasses of different sizes[32]. The experiment time was set to zero when the cadavers had been de-frosted and placed within soil graves. The graves were excavated to approximately 40 cm depth; pigs were placed into metal cages to protect them from the activity of scavengers and then buried at the base of each grave (Supporting Fig. S1). The graves were then completely covered with the grave infill to maintain as much as possible the pre-existing soil conditions, and the various pigs were left to naturally decompose for various PMIs (respectively, one, two, four and six months). Each grave was excavated at the pre-determined time according to the experimental design (P1 after one, P2 after two, P3 after four and P4 after six months PMI). Biological samples were collected simultaneously with soil samples for proteomic analyses that were performed in a separate study (see [30] for bone proteomic analyses performed on these carcasses). Soil samples in contact with the superior part of the carcass were collected from three separate locations, aiming at collecting from within a few centimetres of the remains above the fore-limb area, the abdominal area and the hind-limb area of the body (see Fig. 1 for further details of the sampling locations). These soil samples were then pooled together (i.e., one bag per grave) and three technical replicates were used for the metabarcoding analyses. It should be noted that during each of the excavation and sampling events the weather conditions were always favourable, and for this reason soil sampling was not affected by rainfall or by particular leaching effects. Control soil samples (taken at similar soil depths but without a carcass being present) were collected at locations two and six meters away from the graves. The control samples were obtained concurrently with the last sampling performed in November (six months PMI), to provide a representative record of the microbial activity within the field used for the experiment without microbial contamination from the graves. We are confident that these control samples were taken at a sufficient distance from the graves to exclude any significant influence of carcass-derived bacteria, according to the findings of Singh *et al.*[33]. We assumed that the observable differences within the soil microbial community associated with the close proximity of a decomposing body would be larger and more significant than those caused by variations in local environmental conditions (linked with seasonality, temperature and rainfall), as has been shown by Metcalf *et al.*[28] for different soil habitats, and for this reason our control

samples were collected at different distances from the graves but at a single time point. Soil samples were then placed within individual plastic bags and were frozen immediately at -20°C until the analyses. Overall, we performed metabarcoding analyses on 18 samples (three samples per grave plus six controls). Soil pHs from each sample were obtained by mixing the soil sample with sterile deionised water in a ratio of 1:2.5 (w/v) and measuring the pH of the supernatant water with a pH probe (Mettler Toledo, Cole-Parmer, U.K.) (Table 1). Average daily rainfalls and temperatures have been taken from the local weather station and were reported in Procopio *et al.*[30] as well as soil temperatures that were measured using data loggers (Tinytag Plus 2, Gemini Data Loggers, U.K.)[30].

Samples	C1	C2	P1	P2	P3	P4
Collection site	2 m from graves, 40 cm depth	6 m from graves, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth	In contact with the carcass, 40 cm depth
PMI of the carcasses	-	-	1 month	2 months	4 months	6 months
Body size (kg)	-	-	4.5	6.1	10.8	11.0
Approximate age (weeks)	-	-	3-4	3-4	4-5	4-5
pH	7.3	7.2	7.7	8.1	7.6	7.2

Table 1. Samples used in the experiment with information about sampling location, depth, PMI of the corpses and pH.

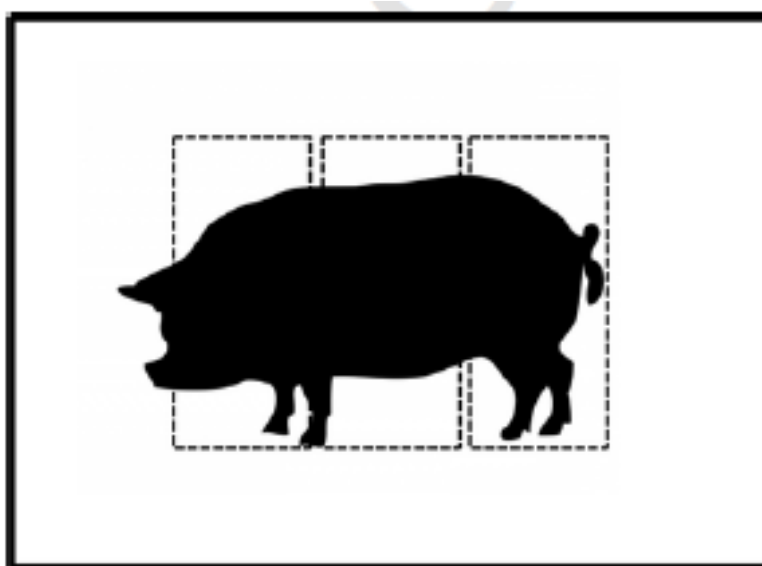


Fig. 1. Illustration of the pig buried in the grave (outer rectangle); dashed rectangles represent the areas sampled in contact with the pig surface (namely, the “fore-limb area” (yellow), the “abdominal area” (green) and the “hind-limb area” (red)).

Chemicals and materials

Primers and agarose gel were purchased from Sigma-Aldrich (U.K.), Platinum Hot Start PCR Master Mix 2x was purchased from Thermo Fisher Scientific (U.K.), Wizard® SV Gel and PCR Clean-UpSystem was purchased from Promega (U.K.), FastDNA® SPIN Kit for Soil was purchased from MP Biomedicals (Europe). All PCRs were performed using a T3000 thermal cycler (Biometra GmbH, Göttingen, Germany).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from 500 mg of soil sample using the FastDNA® SPIN Kit for Soil, following the guidelines provided by the manufacturer and Eubacterial communities were specifically targeted amplifying the 16S rRNA locus[34]. Forward 515FB (GTGYCAGCMGCCGCGGTAA) and reverse 806RB (GGACTACNVGGGTWTCTAAT) primers ([35,36] respectively) were used in combination with overhangs following the guide for 16S metagenomic sequencing library preparation, suggested for the Illumina MiSeq system. Degeneracies were allowed in the primers to decrease eventual biases and to increase the ability to detect organisms characterised by slight modifications in their binding site's sequences. PCR negative controls were run in each analysis to perform a quality check of the amplifications and in all cases these controls gave negative results. PCR reaction mixtures were set up as follows: 12.5 µL master mix (Platinum Hot Start PCR Master Mix 2x), 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM) and 0.5 - 1.5 µL template DNA in a final reaction volume of 25 µL. The thermocycler conditions were set up as follows: denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec and extension at 68°C for 30 sec; final extension at 68°C for 10 min and maintenance of the samples at 4°C. The PCR products obtained were checked on 1.5% (w/v) agarose gels, purified (Wizard® SV Gel and PCR Clean-UpSystem), quantified with Qubit (Qubit Fluorometric Quantitation, Thermo Fisher Scientific, U.K.) and sent to IGA Technology Services (Udine, Italy) for paired-end sequencing using the Illumina MiSeq technology (2 x 250 bp).

DNA data analysis

Paired-end reads from each library were merged using Pear v.0.9.2[37], with the quality score threshold for trimming the low-quality part of a read set at 28, the minimum

length of reads after the trimming process set at 200 bp and the minimum possible length of the assembled sequences set at 200 bp. Unix bash commands were used to select assembled sequences beginning with recognisable forward primer (no mismatch allowed), to trim initial and terminal 19 and 20 bases (corresponding to forward and reverse primers respectively) and to assign a sample specific progressive count to each fragment. Assembled sequences from each library were clustered into Operational Taxonomic Units (OTUs) using a closed reference-based clustering strategy with VSEARCH v2.3.4 (<https://github.com/torognes/vsearch>[38]) at 97% similarity; only clusters encompassing at least 2 sequences were initially retained. The GreenGenes v.13_8[39] was used as a reference for OTU picking and taxonomy assignment. Since not all the entries in the GreenGenes database are defined for the seven conventional taxonomic levels, the missing levels have been filled using the epithet “Unknown” associated with the last available taxonomic level. VSEARCH output was converted into biom format (Biological Observation Matrix format) with biom v2.1.5 program[40] for subsequent statistical analyses.

Statistical Analyses

Numerical ecology statistical analyses were performed within the computing environment R[41] (<https://www.R-project.org/>).

Soil controls were collected from two different locations to better describe the variability of the basal microbiome. DNA from three technical replicates was extracted from each of the two control locations (replicate #1, #2 and #3). During the data analysis, the two control samples collected from the distinct locations were unified arbitrarily into a single control sample, summing up the reads of each of the three replicates (the first two replicates #1 from both soils were unified together to obtain a single “replicate #1” and so for the other two replicates). In other words, we did [replicate 1 + replicate 1]_{C1,C2} + [replicate 2 + replicate 2]_{C1,C2} + [replicate 3 + replicate 3]_{C1,C2} to obtain our three replicates for control samples.

A number of filtering steps were applied to gather the final OTU dataset: OTUs labelled as “mitochondria” (recognised as contaminant in GreenGenes, but not removed from the database) were removed; OTUs with fewer than 50 reads were filtered out as well as samples with fewer than 20 reads and OTUs showing a Coefficient of Variation greater than 3.0 were also removed.

In order to standardize sampling efforts and to allow for comparisons of samples with non-uniform coverage, the OTU table has been normalized by subsampling at even sequencing depth from each sample (38684 sequences per sample) by means of the *rarefy_even_depth* function in the R package PHYLOSEQ v.1.22.3[42]. The rarefaction effects on an OTU table are commonly rendered by means of the rarefaction curves, in order to graphically estimate species richness. Raw species richness counts can only be compared when the species richness has reached a clear asymptote. All the species present in a sample are well described when the curve ascribed to each sample reach its plateau. Rarefaction curves have been rendered by means of the function *ggrare*, provided by the richness.R script from the phyloseq extension package by Mahendra Mariadassou (<https://github.com/mahendra-mariadassou/phyloseq-extended>).

All the taxa abundances have been calculated and graphically plotted with the aim of the R package PHYLOSEQ[42].

Biodiversity analyses was carried out by comparing the richness and evenness of microbial communities of the PMIs. Richness is merely a measure of the number of species in each sample, without taking into account the relative abundance of each species. Evenness, in contrast, takes into account both the number of species, and the relative abundance of each species. Within-sample (Alpha) diversity was assessed by six estimators: "observed number of species", "Chao1", "ACE", "Shannon", "Simpson", "Fisher". The Alpha diversity indices were calculated and plotted by means of the functions *estimate_richness* and *plot_richness* implemented in in the R package PHYLOSEQ[42].

In order to test whether communities were statistically different from each other, a multivariate homogeneity of group dispersions among the different Control and PMI groups was first assessed by means of the *betadisper* and *permutest* (with 999 permutations) functions in the R package VEGAN v.2.5.2[43]. The differences in the composition of bacterial communities in PMIs were rendered by means of a nonmetric multidimensional scaling ordination (NMDS), to visualise Bray-Curtis distances, using the functions *vegdist* and *metaMDS* in the R package VEGAN[43].

The permutational multivariate analysis of variance (PERMANOVA, 999 permutations), as implemented in the *adonis* function of the VEGAN package of R[43], were applied to access whether communities were statistically different from each other.

Indicator species analysis (a classification-based method to measure associations between species and groups of sites[44]) was carried out using the *multipatt* function in the

INDICESPECIES v.1.7.6 R package, with 999 permutations[45] in order to assess whether OTUs (and, if so, which ones) were significantly associated with a particular PMI.

To explore the shifts in the basal communities at different decomposition stages we evaluated which families showed statistically significant differences in abundance at different PMIs applying the Student's t-test with Benjamini and Hochberg False Discovery Rate (FDR) correction[46] with the T.TEST and P.ADJUST functions in R. This generated both *p* values and adjusted *p* values (*q* values), that were used to evaluate the statistical significance of the results reported in the work.

Results and Discussion

Decomposition stages at selected PMIs

One month after burial (pig P1), the carcass was in the putrefaction stage of decomposition but was still recognizable, after two months (pig P2) the carcass was in the putrefaction/liquefaction stage of decomposition, with bones still connected by the ligaments. After four months the carcass P3 was partially skeletonised with still some soft tissues present, and after six months (pig P4) the carcass was fully skeletonised. It has to be noted that the sizes of the carcasses might also have played a role in the decomposition rate of the various animals, as the first two pigs (P1 and P2) were smaller than the last two (Table 1). Notably, bodies with a smaller size are characterised by quicker decomposition than bigger ones[14,15] although we note that the latter studies involved much larger (i.e. order of magnitude or greater) differences in body mass between size categories when compared to the twofold differences in body mass amongst the carcasses in our study. Despite body size variation being a potential confounding factor for the estimation of PMI from carcass decomposition, we believe that this had only a minor impact on our study due to the extended time length of the experiment and the focus on soil microorganisms instead of on the decomposition stage of the animal itself. Samples collected after one month were visually different from samples collected after two and four months, which were characterised by the presence of some adipocere (in particular after two months), that was instead missing in the first grave. We believe that these differences were mostly due to the burial conditions (e.g., moisture, oxygen availability, temperature) and not to the body size of the carcasses (fatter bodies originate more adipocere than smaller bodies, however we have noticed more adipocere in one of the smaller pigs used in the study than in bigger ones). The soil in the fourth grave was

more muddy and slimy, due to the heavy rainfall that introduced non-percolated water within the grave prior to the time of the sample collection.

Metabarcoding analysis of soil bacteria

From the 18 soil samples, a total of 5120369 sequences were collected after libraries' pre-processing: soil controls yielded 758986 sequences (ranging from 36799 to 321857) and PMI soil samples yielded 4361383 sequences (ranging from 51328 to 2326277). Those sequences were clustered with GreenGenes v.13_8, and 3865209 out of 5120369 (75%) found a match at 97% identity with database entries, providing an initial set of 12229 OTUs. After filtering, 9 OTUs were recognized as contaminants ("mitochondria"), 9307 OTUs were removed since their cluster size was smaller than 50 reads, and 90 OTUs were removed because of their Coefficient of Variation being greater than 3.0. A final number of 2823 OTUs was obtained (Supporting Table S1). Rarefaction was performed using the sample showing the lowest OTU abundance, which contained 38684 reads (Supporting Table S2), and rarefaction curves (Supporting Fig. S2) showed that the sequence coverage was adequate for the study and that C1 and P1 were similar each other as well as P2 and P3, as will be discussed further in the text.

A preliminary data analysis inclusive of all samples analysed within this work, performed on the sum of the abundances of the rarefied reads, showed overall that the 99,5% of the microorganisms present within the samples belonged to the Eubacteria kingdom (according to GreenGenes classification). For this reason, all subsequent analyses were done on the Eubacteria fraction only. Within this study, Eubacteria overall were characterised by 2809 different OTUs which are elucidated further below.

Bacterial community changes during progressive stages of decomposition

The first step in the analysis focused on the evaluation of the richness (alpha diversity) of the microbial taxa associated with different PMIs using the Shannon-Wiener index and with boxplots representing the number of species with replicates grouped together around their centroid (Fig. 2). Other indices for alpha diversity such as Chao1, abundance-based coverage estimators, Simpson's and Fisher's index have been also applied, as recommended by Bandeira *et al.*[47], in order to better define the communities (Supporting Fig. S3). Taxonomic richness was roughly constant in all the plots showed in Supporting Fig. S3 between control (C1) and one month post-mortem (P1), with ~2100

different species observed on average (Student's t-test p value = 0.95) but decreased compared to the control by approximately 30% at two months (P2) (p value = 0.0009) and remained lower than the control at four months (P3) (p value = 0.0003) and at six months (P4) (p value = 0.0002) despite a non-significant increase observed starting from P3 onwards. The Shannon-Wiener diversity indices showed statistically significant differences between C1 and P1 (p value = 0.008), C1 and P2 (p value = 0.0004), C1 and P3 (p value < 0.0001) and C1 and P4 (p value < 0.0001).

A decrease in the richness (variability) of the soil microbial population during body decomposition was also observed by Cobaugh *et al.*[26] in soils underneath four human carcasses, in agreement with our findings. Finley *et al.*[29] observed similar results for buried swine carcasses, but the extended time intervals utilised in their study did not allow for a precise comparison with our data. In fact, despite the decrease in species richness that they observed between 0-3 months, they did not collect intermediate data. Furthermore, they demonstrated an increase in taxonomic richness after prolonged PMIs, which is in accordance with our findings. It should also be noted that there are environmental differences between the geographical locations of their experiment and ours (e.g., higher average temperatures and higher humidity at the University of Tennessee ARF than at the HuddersFIELD outdoor taphonomy facility, data from the relevant weather stations are available online respectively at <https://www.weather.gov/mrx/tysclimate> and at <http://www.weatherforce.org.uk/>). These climatic differences may have caused the discrepancies observed here, highlighting the need for additional studies with extended datasets and varying environmental conditions. Due to the potential variations observed here, we are confident that the choice of an U.K. facility for this experiment made this study more applicable to forensic cases in the U.K..

Overall, these results suggest that the presence of the body alters the soil endogenous microbiome resulting in a decrease of the species richness particularly after two months from the burial, at a time period in which the bodies were in their active decomposition stage. The timeline of the progress of decomposition of swine carcasses in shallow burials was also elucidated by Belle *et al.*[48], and their results agree with our findings despite the animals used in their study being older (and heavier) than ours. The availability of a large nutrient resource supplemented by the colonisation of the bacteria from the abdominal cavity altered the original microbial community; the new microorganisms originated by the carcasses appeared to overwhelm the pre-existing community resulting in an overall decrease of the species richness over time. The slight

increase in richness observed during the advanced decomposition stages suggests that after a few months the microbial communities appear to start returning toward original values, but more than six months are required to return to their original state. This observation may be particularly useful in the investigation of clandestine movements of buried carcasses from their original place of burial to another one, despite the absence of the characteristic traits of the vegetation that are instead often used as an indicator for these situations in case of exposed bodies (e.g. presence of cadaveric decomposition islands, CDIs[49]).

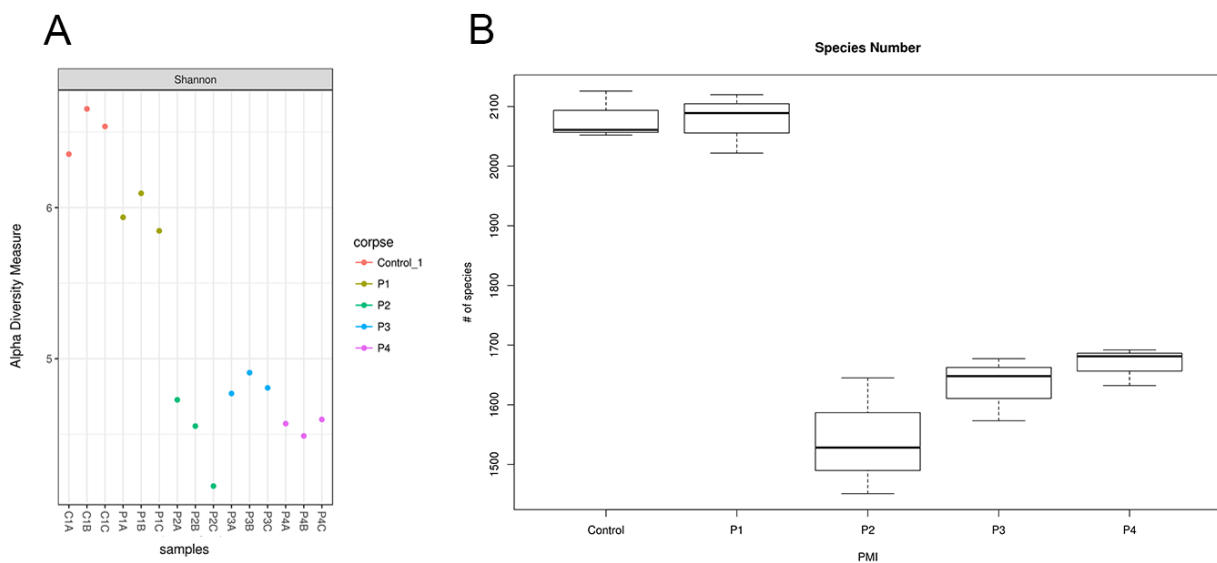


Fig. 2. A) Shannon alpha diversity measure of the samples collected after one (P1), two (P2), four (P3) and six (P4) months post-mortem and B) box plot of the microbial richness of soil samples showing the raw number of species per sample, with standard deviations represented with vertical error bars and medians represented with a bold line in each box.

In order to test whether communities were statistically different from each other, a multivariate homogeneity of group dispersions among the different Control and PMI groups was first assessed by means of the betadisper and permutest (with 9999 permutations) functions in the R package vegan[43] (Fig. 3A) and then assessed by non-metric multidimensional scaling (NMDS), to visualise Bray-Curtis distances (Fig. 3B). The homogeneity of dispersion among groups was supported by a non-significant result in permutest (p value = 0.4732), and it is depicted in a boxplot showing the Bray-Curtis dissimilarity from its centroid (Fig. 3A). Subsequently, PERMANOVA provided significant results ($R^2 = 0.91282$ and $p = 1e-04$), thus indicating that the dissimilarity was influenced by difference in composition between groups. Consequently, we analysed the differences in the taxonomic abundances from different samples to evaluate the beta diversity

between different samples and we used a NMDS plot to visualise Bray Curtis distances (Fig. 3B). Samples collected from the same location clustered together, with P2 being the only one in which the three subsamples did not cluster in a compact way, potentially due to the presence of the adipocere in the soil that may have resulted in a less homogeneous sample. Overall, P1 was closer to the control, whereas samples collected after increased decomposition stages were more distant from it.

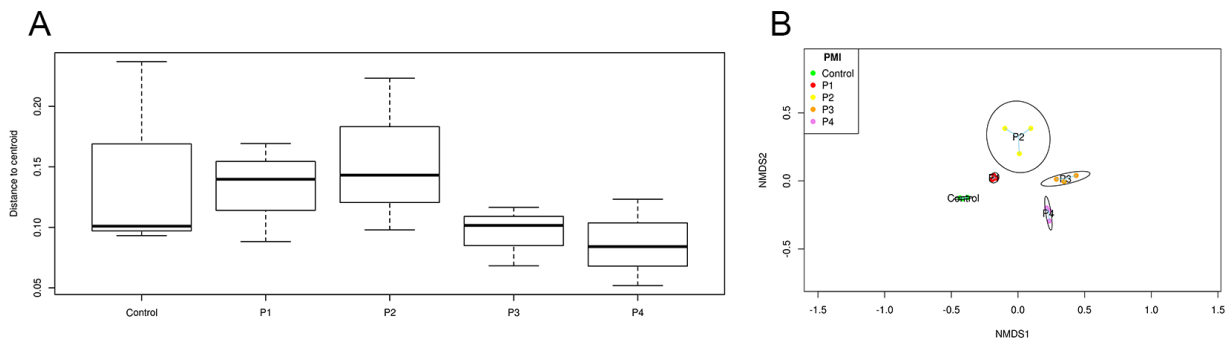


Fig. 3. A) boxplot showing Bray-Curtis dissimilarity of the replicates from the centroid for specific PMIs and B) NMDS plot showing an increase in beta diversity between the control soil and the gravesoil, with increasing variability after prolonged PMIs compared with shorter ones represented by expanded ellipses for P2, P3 and P4 (P1 = 1 month, P2 = 2 months, P3 = 4 months, P4 = 6 months). Standard deviation is represented by the dimension of the ellipses.

In order to characterise and identify which were the major differences observed between the different groups, we initially focused on the abundances of phyla. Overall, Proteobacteria were the most abundant phylum followed by Bacteroidetes, then by Acidobacteria which were almost as abundant as Actinobacteria, and by Firmicutes (Supporting Fig. S4). Comparing the different PMIs, specific trends were observed at specific decomposition stages (Fig. 4); in particular, Proteobacteria increased in their abundance from P1 to P4 (with a slight decrease in P3) and were the dominant phylum at the first time point analysed here but also after two and six months post-mortem. Bacteroidetes also increased with increasing PMIs, reaching their maximum after four months PMI and overcoming the abundance of Proteobacteria at this specific time point. Finally, Firmicutes abundances increased during the later stages of decomposition. Control soil was dominated by Acidobacteria followed by Proteobacteria. Acidobacteria were the only phylum for which abundance decreased constantly after the introduction of the carcasses in the soil, with decreasing levels from one to four months and with a partial recovery after six months. Some other phyla showed a similar behaviour to this latter example, with decreasing abundances accompanying with increasing PMIs compared with

the control soil (Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia).

The observation that Proteobacteria are the most abundant phylum within the majority of the experimental samples analysed here is in accordance with previous studies[12,27,29,50]. Finley *et al.*[29] found Proteobacteria, Acidobacteria and Actinobacteria to be the most abundant phyla in all samples, similar to what was found here, even though they found lower levels of Bacteroidetes in comparison with our study (Fig. 4). Similar observations for Acidobacteria, Nitrospirae and Verrocumicrobia were obtained by Cobaugh *et al.*[26], where the relative abundances of these phyla decreased to less than 15% of their abundances in the control samples during later stages of decomposition. The increase of Bacteroidetes has not previously been shown in the literature, despite the fact that here they reached high abundances over the course of our experiment. On the contrary, the increase of Firmicutes was already documented in previous works; Metcalf *et al.*[27] showed that Firmicutes increased with prolonged PMIs and Pechal *et al.*[12] found Firmicutes to be the most abundant phylum at prolonged decomposition stages, in contrast with our findings (namely that Proteobacteria dominated the later decomposition stages). However, the results for Firmicutes reported by Pechal *et al.*[12] could be due to the presence of insects, particularly flies, accessing the carcass. Another study performed by Pechal *et al.*[51] on swine decomposition, which excluded the exposure of the carcasses to flies, in fact showed that Proteobacteria remained the most abundant phylum after prolonged decomposition stages. Therefore, it is likely that our experimental design excluded access by insects such as flies to our carcasses, and this may explain the reduced abundance of Firmicutes observed here in contrast with the previously mentioned findings obtained on exposed remains.

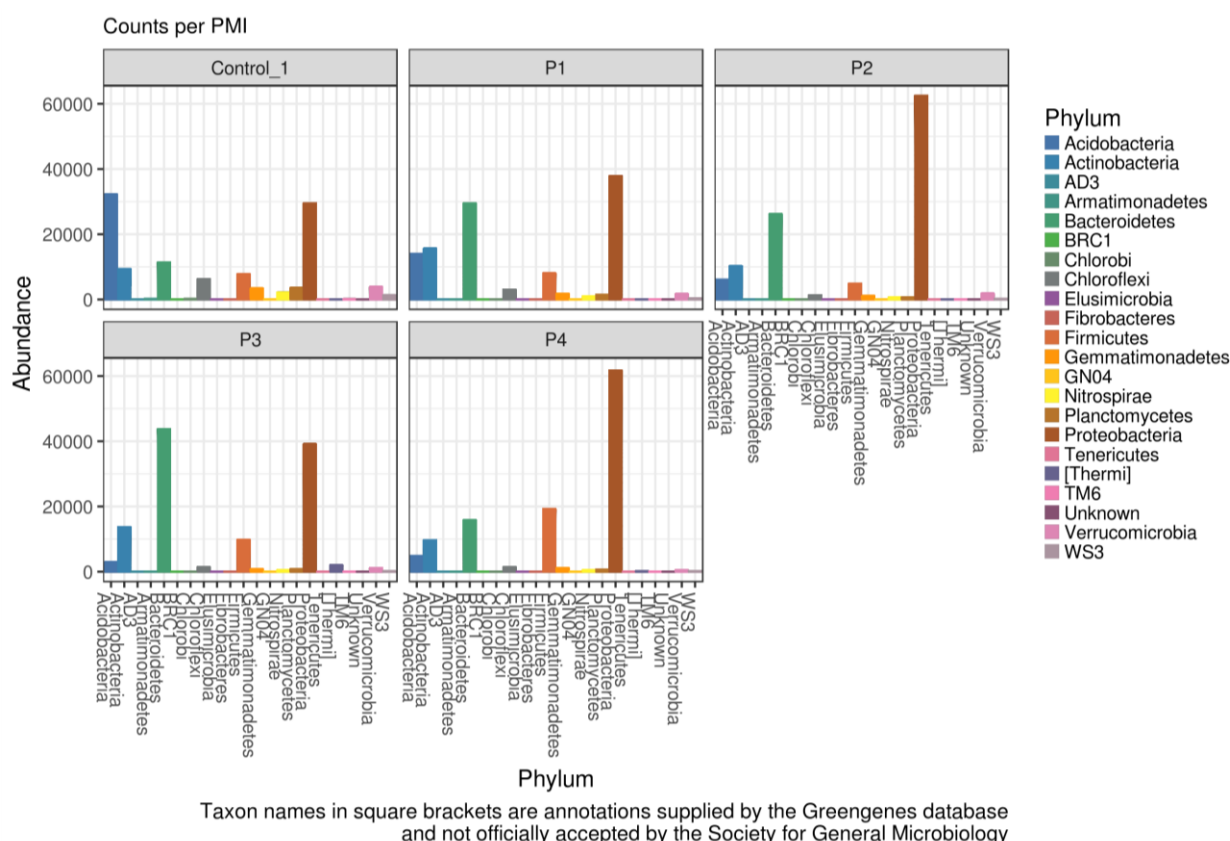


Fig. 4. Bar chart with abundances of taxa at a phylum level associated with control soil and with the experimental samples.

The pH of the soil associated with the graves increased from 7.2 in the controls to 8.1 after two months PMI and decreased again over the course of the experiment returning to the starting value after six months (Table 1). This finding is in contrast to the results of Coughlin *et al.*[26] but is supported by other studies[23,28,52,53][23,52,53]. The presence of a linkage between the alkalinity of the soil and the formation of adipocere proposed by Forbes[54] and by Olakanye *et al.*[23] found support in this study, where the most alkaline soil contained the greatest amount of adipocere. The increase in gravesoil pH is associated with the active decay of the carcass, and in particular with the rupture of the skin and the release of ammonia-rich nutrients within the soil. Data obtained here suggest that the rupture of the corpses started approximately after one month from the deposition of the carcasses but the release of the nutrients reached its maximum only after two months, supporting also our findings about the taxonomic richness of the samples previously discussed. This finding correlates well with other studies on Acidobacteria associated with decomposing carcasses[27,55]. Acidobacteria is a phylum of bacteria characterised by different subgroups adapted to survive in various pH conditions[56], with some groups preferring basic pH and others preferring acidic conditions. However, the groups which here showed the most significant changes between control and gravesoil

were groups III and V [57] (families iii1-15 and RB41 respectively, see Fig. 5 for reference), and these groups are adapted to survive better with low pHs and worse with high pHs[56]. This explains the shift in the Acidobacteria populations observed with increasing pHs in this study. Acidobacteria abundances started to increase again after six months, even though the pH started to be more favourable after four months; this may be related to their slow growth rate in comparison with other bacteria[58]. Additionally, Acidobacteria are oligotrophic bacteria[55], which means that they prefer environments with low levels of nutrients in contrast with copiotrophic bacteria which prefer environmental conditions rich in nutrients. Their recurrence after six months indicates from a microbial point of view the almost complete consumption of the organic matter that was introduced with the carcass, as was visually confirmed by the quite complete skeletonization of the body recovered after six months.

For a more detailed description we examined variation at the family level (Table 2 and Fig. 5). Within the phylum of Proteobacteria, one of the most significant variations between control and gravesoil was the increase of the Pseudomonadaceae members, particularly after two months. Also, Xanthomonadaceae increased notably in the gravesoil samples compared with the control, with larger variations especially recorded after one and six months post-mortem. Comamonadaceae showed an increased abundance in particular after six months, despite being already relatively abundant in the control. Caulobacteraceae, on the contrary, increased particularly at the first time-point and decreased during the advanced decomposition stages. Finally, Alcaligenaceae increased continuously with increasing PMIs. Focusing on Bacteroidetes instead, we found members of the Flavobacteriaceae family which increased after one, two and particularly after four months, and then were reduced (despite remaining more abundant than in the control) after six months. Sphingobacteriaceae were particularly abundant after the first month and then decreased progressively reaching almost basal levels after six months, leaving space for the arrival of Porphyromonadaceae (that were almost absent in the control) in particular from two months onwards. Saprospiraceae, a family of bacteria present in the control, decreased after the first month and then almost disappeared after prolonged PMIs. Finally, within the Firmicutes phylum, we noticed variations in the abundance of Paenibacillaceae between control and gravesoil, with the samples collected at the last time point being the sample with the greater abundances of bacteria belonging to this family in comparison with other samples. Despite the decline of some copiotrophic taxa after six months and the partial re-establishment of the initial conditions within the soil microbiota, our results

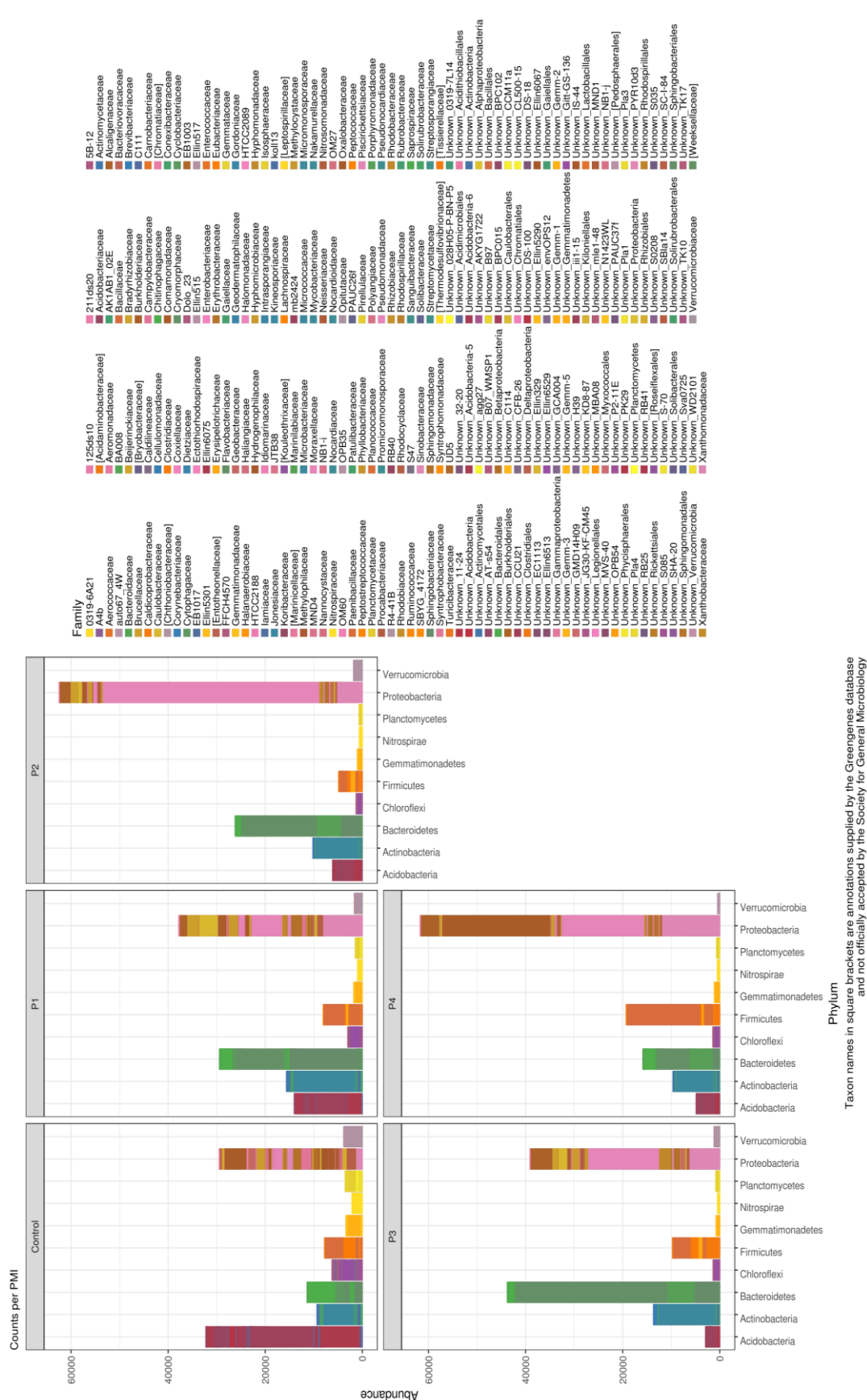
suggest that six months are not sufficient to completely recover the starting conditions in this burial environment. Some phyla far from their complete repopulation within the soil, such as Acidobacteria, Chloroflexi, Gemmatimonadetes, Nitrospirae, Planctomycetes and Verrucomicrobia (Fig. 5) and several taxa specifically associated with the presence of the pig carcass were still persistent after six months, as described in the following section of the paper.

Several of these families were already known to be associated with decomposition studies; for example, Cobaugh *et al.*[26] showed slight increases of Xanthomonadales during the initial decomposition stages, and a rapid increase in their abundances during the advanced decay stages. Metcalf *et al.*[27] showed that Alcaligenaceae increased in the gravesoil under exposed mouse remains during their advanced decay stage of decomposition, in accordance with our findings here. However, they showed also that Sphingobacteriaceae increased during the advanced decay stage of decomposition[27], in contrast with the results presented here.

Overall, the results obtained here showed the existence of several families in which abundances increase or decrease at specific decomposition time stages in a statistically significant way, although these patterns were sometimes in accordance with other studies and sometimes not. The presence of very different experimental designs across all the studies cited here (including humans, pigs and mice, exposed or buried, within different types of soil and different environmental conditions) makes the interpretation of the results more challenging. However, the similarities found across these different works show the potential and the importance of the research in this field allowing the identification of specific signatures that may be promising for forensic scenarios.

Phylum	Firmicutes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Bacteroidetes	Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria	Proteobacteria
Families	Paenibacillaceae	Saprospiraceae	Porphyromonadaceae	Sphingobacteriaceae	Flavobacteriaceae	Alcaligenaceae	Comamonadaceae	Caulobacteraceae	Xanthomonadaceae	Pseudomonadaceae		
C1	780	930	11	1075	1096	164	4575	171	1393	1765		
P1	157	432	831	14132	9466	1508	1749	3856	8186	6287		
P2	87	100	4431	3965	14527	2203	968	782	5313	44565		
P3	79	133	5082	5202	30906	4480	696	1837	6352	14556		
P4	67	90	4360	1437	6266	3759	22242	194	12006	16885		
ANOVA p value	0.048	< 0.0001	0.0008	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001		
C1 vs P1 q value	0.0235	0.0154	> 0.05	< 0.0001	0.0002	0.0003	0.0119	< 0.0001	0.0003	0.0145		
C1 vs P2 q value	0.0187	0.0005	0.0004	0.0002	< 0.0001	< 0.0001	0.0038	0.0013	0.0069	< 0.0001		
C1 vs P3 q value	0.0187	0.0005	0.0004	< 0.0001	< 0.0001	< 0.0001	0.0036	< 0.0001	0.0021	< 0.0001		
C1 vs P4 q value	0.0187	0.0005	0.0004	> 0.05	0.0037	< 0.0001	< 0.0001	> 0.05	< 0.0001	< 0.0001		

Table 2. Abundances of the families which showed an increased beta variability between different samples with ANOVA p values and with false discovery rate (FDR) correction (Benjamini, Krieger and Yekutieli) q values.



Identification of endogenous mammalian microbial communities and basal communities

Decomposing mammal carcasses contain specific microbial communities associated with their skin and within their gut, and the identification of some of these specific mammalian microbes within the soil may have important forensic relevance. In fact, it would be interesting to make comparisons with studies undertaken on human corpses to evaluate the extent of the suitability of swine bodies in simulating human burials, as we might expect commensal biota to differ in pigs and humans for several reasons, including differences in diet, human consumption of drugs such as antibiotics, levels of hygiene, differences in immune systems, etc. Also, it may be important to understand which of the basal microbes also present within the control samples varies depending on the decomposition stage, despite the presence of microbes introduced by the carcass. We wanted to test various research questions, such as are pigs suitable as proxies for humans? Do the control microbiota change after cadaver burial? Furthermore, we also wanted to assess if the variations observed between the control soil and the gravesoils were due to the introduction of new bacterial species from the mammals and/or to the colonisation of the carcass by new bacterial species attracted by the decomposing bodies which were not present in the control soils. For this reason, we excluded all the OTUs previously identified within the control keeping only OTUs which were exclusively present within the gravesoil (Supporting Table S3) or OTUs that characterised the basal microbiome (e.g. excluding the OTUs unique for gravesoil) (Supporting Table S4). In summary, we found 2331 different OTUs present within the basal microbiome and 478 taxa specifically associated with the gravesoil only (not identified in the control).

The microbiome associated exclusively with the presence of the carcass showed a wide variety of different families (Fig. 6), with several of them being unique for the gravesoil and never being identified in the control soil (Table 3).

Families uniquely present in gravesoil	
Acholeplasmataceae	Enterococcaceae
Actinomycetaceae	Gordoniaceae
Bacteroidaceae	Lachnospiraceae
Campylobacteraceae	Neisseriaceae
Dietziaceae	Ruminococcaceae

Table 3. List of families identified uniquely in gravesoil and not in control soil.

Indicator species analysis was undertaken to investigate the presence of specific species associated with the basal microbiome, or with the presence of the carcasses (significance level = 0.05), showed that overall 873 species contributed to the differences observed between the control and the gravesoil (p values ≤ 0.05) and 187 species contributed significantly to discriminate mammals-associated bacteria from basal microbes (Supporting Table S5).

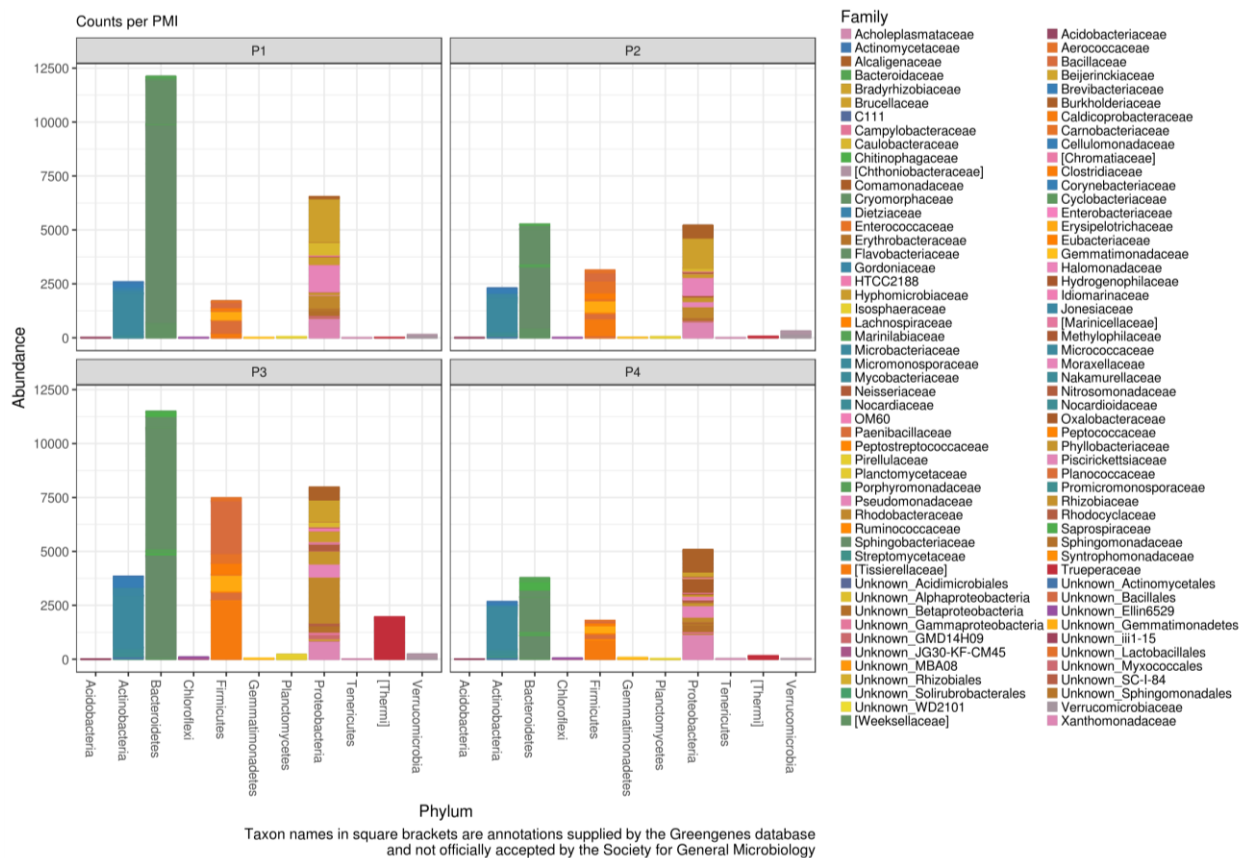


Fig. 6. Microbiome associated uniquely with the mammalian carcasses and not identified within the control sample. Abundances per each OTU have been reported into families (see legend) and aggregated into phyla (X axis).

Interestingly, only Bacteroidaceae have been previously mentioned in the literature in studies of the decomposition of carcasses in the soil. In particular, members of this family have been previously found in the abdominal cavity during the bloating stage of decomposing mice[27]. After the rupture of the corpse, they decreased dramatically within the body and increased in the soil underneath; we found here that their survival within the gravesoil lasts at least for six months from the deposition of the body (Supporting Table S2) in particular for *Bacteroides*. This particular finding is in accordance with Cobaugh *et al.*[26] and proves that non-endogenous soil species can survive for prolonged times outside their original environment (e.g., gut) probably due to the reduced concentrations of

oxygen that characterise the burial environment that accommodates the presence of anaerobic bacteria such as *Bacteroides*.

It has to be noted that during this experiment we did not have control on the freezing procedure adopted prior to the beginning of the burials (and in particular on the period of time the pigs had been kept frozen) due to the previously mentioned ethical and legal reasons. Although we are aware that this procedure may have had an impact on the survival of gut bacteria, a study done on frozen foods showed that microorganisms can survive during freezing, storage and thawing[59] and more recently there has been evidence for the repair of eventual DNA lesions in bacteria at -15 °C, resulting in an overall survival and functionality of microbes found in icy environments[60]. Our results indicated the existence of several taxa specifically associated with the carcass, supporting the idea that the freezing procedure did not completely kill the microbiome associated with pigs. Moreover, our findings on the presence of Bacteroidaceae uniquely in the soil with the carcasses and not in the control further confirms this hypothesis, indicating that the freezing procedure did not significantly affect the survival of gut microbes.

To explore the shifts in the basal communities at different decomposition stages we evaluated which families showed statistically significant differences in abundance at different PMIs (Student's t-test with Benjamini and Hochberg False Discovery Rate (FDR) correction, Supporting Fig. S5-S8 and Supporting Table S6). This particular analysis may be useful to look for more generic biomarkers that may be applicable to a broader range of forensic contexts in a more general way (e.g. not only to pigs but also to humans), excluding specific bacteria that could have been brought by the animal and that could suffer from individual variability.

The highest statistical power was associated with Geobacteraceae, which were significantly less abundant in all the gravesoil samples in comparison with the control soil. Additionally, other significant variations were observed at the first time point with a decrease in the abundance of Phycisphaerales, at the second time point with an increase of Alcaligenaceae and a decrease of Cytophagaceae, and at the third time point with an increase of Flavobacteriaceae and a decrease of Cytophagaceae, Gemmataceae, Geobacteraceae, Marinicellaceae, Piscirickettsiaceae and Planctomycetaceae (Supporting Table S6).

Despite the general trend of decline of basal species observed within the gravesoil, we identified an interesting opposite trend for Alcaligenaceae and Flavobacteriaceae (Supporting Table S6 and Fig. 7). Alcaligenaceae belong to the class of β -Proteobacteria

and were shown to increase in abundance during advanced decomposition stages in the soil underneath decomposing mice[27]. This is consistent with our results and the idea that, regardless of the type of corpse used for the study and the type of soil, members of a microbial family may become in future useful targets to estimate the PMI of the victim or to infer the former presence of bodies in an advanced state of decomposition which have been moved from the burial place to a separate location. Flavobacteriaceae, that belongs to the phylum Bacteroidetes, on the contrary has never been found to be correlated with decomposition studies, and further work on larger datasets may clarify their role during the active decay stage of buried carcasses to exclude external effects (e.g. seasonality, humidity, soil type) on the recorded increase in their abundances over time.

In addition to the most significant families that were differentially abundant in different samples, other families deserve to be mentioned here due to their statistical significance. In particular, a significant increase in the Porphyromonadaceae (Bacteroidetes phylum) was observed during all the phases of the decomposition from two months onwards, Bacillaceae (Firmicutes phylum) increased particularly after six months, Pseudomonadaceae (Proteobacteria phylum) increased after two months and remained quite abundant thereafter, and Comamonadaceae (Proteobacteria phylum) increased after six months (Fig. 7).

Taken together, these results suggest that basal soil microbes, in addition to the mammalian-associated microorganisms, can contribute to the identification of specific biomarkers that can assist scientists in the evaluation of the decomposition grade of mammalian corpses. More specifically the analysis of combined basal and introduced biota could avoid some biases introduced by the inter-individual variability that potentially may exist between different carcasses and that may further complicate the interpretation of the results. In this regard, an improved experimental design should consider more bodies for each phase of decomposition, in addition to suitable soil controls at each sampling site in order to explore and subtract the biodiversity associated to a given site at a given time.

Conclusions

In this study we have shown that shifts in the microbial populations within the gravesoil collected from experimental shallow burials show trends comparable with other studies, despite the differences existing within the experimental designs used, among species and within singular individuals, demonstrating the great potential of this approach for forensic research. We were able to show a general reduction in taxonomic richness of soil microbes with increasing PMIs, in accordance with other studies. We have also provided the first substantial characterisation of specific taxa associated with gravesoil collected from burial environments, focusing on mammals-associated microbes and on basal communities to overcome the limits due to inter-individual and inter-species variability. We note that this work is only exploratory, and for this reason the results showed here would benefit from further studies in order to be corroborated and validated. However, despite not being able to reach unique and exhaustive conclusions about specific biomarkers that can be uniquely associated with specific PMIs, this study showed promising results that will help to frame the design of studies in the future. It is possible that further bioinformatics investigation, such as through the application of Random Forest models[27], may also be useful in further interrogation of this dataset to discover if the changes observed here may be useful to estimate the PMI of the bodies, or if they compare well with the predictive models already proposed by Metcalf *et al.*[27,28] for exposed corpses.

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